

## REMARKS

With this amendment, claims 1-28 are pending. No new matter has been added with this amendment.

### Drawings

The Office Action indicates that the present application was filed with informal drawings that are acceptable for examination purposes only and that correction is required. Applicants note that 9 pages of formal drawings (Figures 1-8) were filed in the USPTO on 6 August 2001 in response to a Notice to File Corrected Application Papers. Applicants received notice of USPTO receipt of these drawings by return post card. Applicants therefore believe that the requirement for correction of informalities in the drawings should be withdrawn.

### Amendments to the Claims

Claim 1 has been amended to delete the phrase 'can be' and replace it with the word "is". This amendment was made to clarify that the reagent of the applicant's invention is isotopically labeled.

Claim 1 has been amended to delete the second occurrence of the phrase "that selectively". This deletion was made to correct a typographical error.

Claim 1 has been amended to eliminate the limitation "certain" which is believed to be unnecessary. Support for this amendment is found at page 6, line 8.

Claim 6 has been amended to: replace the word "an" before "affinity label" with "the"; replace the word "a" before "protein reactive group" with "the"; and replace the word "a" before "linker group" with "the". All of these amendments were made to correct antecedent basis.

Claim 6 at lines 8-9 was amended to insert the phrase "the group consisting of" before "O" and the word "and" before "aryl". This change was made to correct the Markush language.

Claim 6 at lines 11 and 12 was amended to insert the phrase “the group consisting of” before “COO” and to replace the word “or” with “and”. This change was made to correct the Markush language.

Claim 8 was amended to replace the word “or” with “and”. This change was made to correct the Markush language.

Claim 13 was amended to replace the phrase “and a” with “and an” and correct a grammatical error.

Claims 15 and 16 were amended to change the phrase “the deficiency” to “a deficiency”. These changes were made to correct antecedent basis.

Claim 17 was amended to add the phrase “acid sphingomyelinase, galactocerebroside  $\beta$ -galactosidase,” to more fully claim the Applicants’ invention. Support for this amendment is found on page 34, lines 22-26.

Claim 18 was amended to add superscripts ( $B^1$  and  $B^2$ ) to maintain consistency with claim 1.

#### Amendments to the Sequence ID List

Sequence ID numbers 60-64 were amended to be consistent with the sequences in Fig. 4B. The original sequence ID list inadvertently omitted the first amino acid in each peptide. Substitute sheets for the paper copy and a substitute copy of the computer-readable form of the sequence listing are submitted herewith. The substitute copy in computer readable form is the same as the substitute paper copy.

#### Amendments to the Specification

The specification has been amended to correct obvious typographic and clerical errors including spelling errors. The specification at pages 22 has been corrected to refer to the correct figures which is clear from the context of the specification.

Applicants have inserted a Brief Description of the Figures as requested by the Examiner. This amendment is fully supported in the specification which refers to the figures.

Applicants note that both a Summary of the Invention (beginning at page 4) and a Detailed Description of the Invention (beginning at page 11) are present in the as-filed application. It is believed that the Examiner's requirement for correction was made in error and no amendment has been made to address this requirement.

#### Amendments to the Schemes

Portions of Schemes 3, 5, 14, 15, 16, 18, 20, 21, 22, and 23 were amended to restore consistency with the schemes as-filed, and to correct inadvertent errors introduced with the submission of the replacement schemes. No new matter is introduced by the amendment.

#### Amendments to the Drawings

Figure 4B was amended to change "L", first occurrence in peptide 3, to "I". This change was made to maintain consistency with the as-filed drawings and correct an error introduced during the preparation of formal drawings.

Figures 5B ad 5C were amended to correctly identify the peptide sequences for ADH1 and ADH2 as identified in Table 3.

Applicants respectfully request approval of the proposed drawing corrections.

#### Information Disclosure Statement

With respect to the Information Disclosure Statement filed November 30, 2001 (received by the United States Patent and Trademark Office on January 14, 2002), Applicants note that all of the references cited in the form 1449 filed with the Information Disclosure Statement were cited in parent U.S. application 09/383,063 from which the present application takes priority. Applicants are not required to submit copies of references that were provided in a parent application.

Applicants request that the Examiner refer to, and consider, the copies of cited references filed in the parent application and provide Applicants with a copy of the form 1449 indicating that the references were considered.

#### Rejections under 35 USC § 112

Claims 1-28 were rejected under 35 U.S.C. § 112, second paragraph as allegedly indefinite for failing to point out and distinctly claim the subject matter regarded by the Applicants as their invention. Claim 1 has been amended to delete the word "can" and replace it with "is". Claim 1 has also been amended to delete the second occurrence of the phrase "that selectively". Claim 1 has also been amended to change the phrase "certain protein functional groups" to "a protein functional group". Claim 6 has been amended to correct both antecedent basis and Markush group language. Claims 15 and 16 have been amended to correct antecedent basis.

In view of all of the foregoing, Applicants respectfully request withdrawal of all of the rejections under section 112.

#### Rejections under 35 USC § 103

In the Office Action, claims 1-7, 9-10, 12-16, 18-20 and 22-28 were rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Sigler et al. (U.S. 4,798,795) in view of Duncan et al. (Analytical Chemistry, March 1998). Applicants respectfully traverse this rejection.

Applicants claim a protein reactive, isotopically labeled affinity reagent for mass spectrometric analysis of proteins that comprises an affinity label specific for binding to a capture reagent, a protein reactive group that selectively reacts with a protein functional group, and an isotopically labeled linker group joining the two. Applicants also claim a reagent kit comprising the above reagent.

The Office Action states that Sigler et al. discloses an affinity tagged protein reactive reagent for use in isolating proteins in a sample mixture, the reagent comprising an affinity label, a disulfide linker group, and a thiol reactive group. The reagent reacts with a thiol-containing protein to form a biotin-dithio-linked protein. The Office Action further states that reagent may then be captured using an avidin affinity matrix to selectively bind biotin, and then released from the affinity matrix by reduction of the disulfide bonds. The Office Action states that Sigler et al. fails to teach differentially and isotopically labeling the proteins and peptides using stable isotopes.

The Office Action also states that Duncan et al. teaches analyzing peptides and proteins on the femtomole scale using isotope dilution chromatography and mass spectrometry, and further teaches the addition to sample mixtures of stable isotope labeled amino acids as internal standards. The Office Action further states that Duncan et al. use stable isotope labeled standards for each amino acid to reduce the need for correcting for losses of specific protein components to provide accurate and sensitive quantification of proteins and peptides.

The Office Action states that it would have been obvious to one of ordinary skill in the art at the time of the present invention to have incorporated the stable isotopes taught by Duncan et al. into the reagent of Sigler et al. because Duncan et al. specifically used the stable isotope to label internal standard proteins and peptides in a chromatographic/MS system and that one of ordinary skill in the art would have been motivated to combine the teachings of Duncan et al. and Sigler et al. because Duncan specifically taught the advantage of using stable isotope labeled standards in protein/peptide quantitation methods.

Applicants argue that the reagent of Sigler et al. has been mischaracterized in the Office Action. The reagent of Sigler et al. is designed to ultimately release free, unlabeled protein from the affinity matrix to which the protein is bound through biotin (column 1, line 40 – column 2, line 32). Specifically, thiol containing proteins are biotinylated through a disulfide exchange reaction between the reagent and the protein (see the reactive group at the far right of Formula I of Sigler et al.). After capture on an avidin affinity matrix, the dithiol bond is ruptured between the reagent and the thiol-containing amino acid of the protein, and the free, unlabeled protein is released.

Applicants further argue that the teachings of Duncan et al. have also been mischaracterized in the Office Action. First, Duncan et al. teaches the isotopic labeling of amino acids, *not* proteins and peptides as stated in the Office Action. The isotopically labeled amino acids are used as internal standards for amino acid composition analysis (AAA). Second, the internal standard amino acids of Duncan et al. are labeled by the replacement of C or H with stable isotopically heavy C and H, and not by the use of an additional *reagent* having a reactive group to react with amino acids. Third, the internal standards of Duncan et al. are used to identify a single, isolated and purified protein, and not for quantitation of protein/peptide mixtures as stated in the Office Action. Duncan et al. teaches using isotopically labeled amino acid *internal standards*, which are *not* the equivalent of isotopically labeled affinity reagents for

labeling proteins. Nothing in Duncan et al. teaches or suggests the use of such an affinity reagent.

Applicants also argue that a person of ordinary skill in the art would have no motivation to use an isotopically labeled internal standard in combination with the protein purification reagent of Sigler et al. because Sigler et al. teaches and suggests only the isolation of proteins from a sample, and *not* quantitation of proteins. Applicants argue that 1) it would not have been obvious to one of ordinary skill in the art to utilize the internal standard amino acids of Duncan et al. in the purification reagent of Sigler et al. because the purification reagent is ultimately removed from the protein and 2) nothing in either Sigler et al. or Duncan et al. suggests or teaches that proteins and peptides could be quantitated using mass spectrometry of a labeled protein or peptide.

Applicants also argue that the combination of the isotopically labeled internal standard of Duncan et al. with the affinity purification reagent of Sigler et al., does not teach an isotopically labeled affinity binding reagent, as is claimed in the present invention.

Because this rejection is based at least in part on inaccurate characterizations of the teachings of Duncan et al. and Sigler et al., because Duncan et al. does not teach the use of an isotopically labeled affinity reagent, because there is no motivation to use any type of isotopically labeled internal standards in the protein purification method of Sigler et al., and because the combination of these two references does not provide the Applicants' reagent as now claimed in claims 1-7, 9-10, 12-16, 18-20 and 22-28, Applicants submit that no *prima facie* case of obvious has been made, and request that this rejection be withdrawn.

In the Office Action, claim 21 was rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Sigler et al. (U.S. 4,798,795) in view of Duncan et al. (Analytical Chemistry, March 1998) as applied to claims 1-20 and 22-28 and in further view of Allen et al. (U.S. 5,438,017). Applicants respectfully traverse this rejection.

Claim 21 is directed to the reagent of claim 1, A-L-PRG, wherein the linker is isotopically labeled by substitution of any linker atom with its heavy isotope.

The Office Action states that Allen et al. disclose the addition of internal standards of proteins and peptides that are isotopically labeled with heavy metal isotopes (deuterium) to samples for analyzing proteins and peptides using a GC/MS system.

Applicants first note that Sigler et al. and Duncan et al. are applied in the previous rejection to claims 1-7, 9-10, 12-16, 18-20 and 22-28, and not to claims 1-20 and 22-28 as stated above. Applicants request clarification as to which claims are allegedly rejected in view of Sigler et al. and Duncan et al.

As argued above, the combination of Sigler et al. and Duncan et al. does not constitute a *prima facie* case of obviousness of claim 1. Allen et al. only teaches another example of using a known internal standard to assay for the presence of a known compound (sulfhydryl amino acid or methylmalonic acid) and does not cure the deficiencies of the Sigler-Duncan combination. Specifically, Allen et al. teaches the use of deuterated internal standards, which are most likely prepared by the replacement of hydrogen in, for example, methylmalonic acid, with deuterium. Allen et al. *does not* teach or suggest the use of an isotopically labeled reagent that reacts specifically with *sample* compounds, e.g. sulfhydryl amino acid or methylmalonic acid. As argued above regarding Duncan et al., isotopically labeled internal standards such as those taught by Allen et al. are *not* the equivalent of isotopically labeled affinity reagents for labeling *sample* compounds of interest.

Applicants also argue that the teachings of Allen et al. have been mischaracterized in the Office Action as related to the analysis of proteins and peptides. Allen et al. relates to the analysis of known compounds (sulfhydryl amino acids or methylmalonic acid) which *are not* proteins or peptides. In addition, Applicants note that Allen et al. and Duncan et al. *both* teach the use of deuterium (a heavy isotope of hydrogen) to label internal standards and as a result, Allen et al. does not add to the teachings of Duncan et al. with respect to the use of heavy isotope labels. Consequently, and in view of the differing objectives of the methods of Sigler et al., Duncan et al., and Allen et al., and the reagents used to practice these methods, there is no motivation to combine their teachings. As argued above, even if the combination of Allen et al. with Sigler et al. and Duncan et al. were motivated, if practiced the combination would not provide the isotopically labeled protein reactive affinity reagent as now claimed.

In view of the foregoing, Applicants submit that no *prima facie* case of obviousness has been presented and as a result this rejection is improper and should be withdrawn.

In the Office Action, claims 8, 11 and 17 were rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Sigler et al. (U.S. 4,798,795) in view of Duncan et al. (Analytical Chemistry, March 1998) as applied to claims 1-7, 9-10, 12-16, 18-20 and 22-28 and in further view of Markert Hahn et al. (U.S. 5,514,559). Claims 8, 11 and 17 all depend from claim 1. Applicants respectfully traverse this rejection.

Claims 8, 11, and 17 provide specific affinity labels and protein reactive groups for the isotopically labeled reagent of claim 1.

The Office Action states that Sigler et al. and Duncan et al. fail to teach the affinity groups and protein reactive groups claimed in claims 8, 11 and 17. The Office Action also states that Markert-Hahn et al. discloses stable reagents comprising an affinity label, a linker, and protein reactive groups combined as immunologically active conjugates for use in binding interactions between proteins, enzymes, peptides, haptens and polymers.

Markert-Hahn et al. discloses the use of affinity labels known in the art as part of affinity linkers that enable two different substances to be coupled stably. Markert-Hahn, et al., uses two trivalent linkers each having an arm to bind to a substance of interest, and two arms to form a dithio bond between them. Markert-Hahn et al. does *not* teach or suggest the isotopic labeling of an affinity label linked to a protein reactive group. Applicants argue, therefore, that the linkers of Markert-Hahn et al. do not cure the deficiencies of the Sigler-Duncan combination, and if the Markert-Hahn linkers are combined with Sigler et al. and Duncan et al., they do not teach the claimed reagent.

Because the combination of Markert-Hahn et al. with Sigler et al. and Duncan et al. does not present a *prima facie* case of obviousness, the rejection is improper and should be withdrawn.

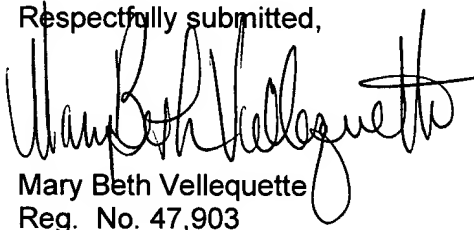


## CONCLUSION

All rejections and objections of the claims are believed overcome. Reconsideration and withdrawal of the rejections and objections is respectfully requested.

With this amendment, this application is believed to be in order for allowance and passage to issuance is respectfully requested. It is believed that no fee is due with the submission of this Amendment. If this is incorrect, however, please charge the required fee and the fee for any extension of time needed to Deposit Account No. 07-1969.

Respectfully submitted,

A handwritten signature in black ink, appearing to read "Mary Beth Vellequette", written over the typed name and registration number.

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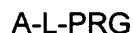
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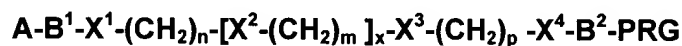
Amended Claims - Version with markings to show changes made.

1. (Once amended) A reagent for mass spectrometric analysis of proteins which has the general formula:



where A is an affinity label that selectively binds to a capture reagent, L is a linker group which [can be] is differentially labelled with stable isotopes and PRG is a protein reactive group that selectively [that selectively] reacts with [certain] protein functional groups.

6. (Once amended) The reagent of claim 1 which has the general formula:



where: A is [an] the affinity label;

PRG is [a] the protein reactive group; and

$[B^1-X^1-(CH_2)_n-[X^2-(CH_2)_m]_x-X^3-(CH_2)_p-X^4-B^2]$   $B^1-X^1-(CH_2)_n-[X^2-(CH_2)_m]_x-X^3-(CH_2)_p-X^4-B^2$   
is [a] the linker group wherein:

$X^1$ ,  $X^2$ ,  $X^3$  and  $X^4$ , independently of one another, and  $X^2$  independently of other  $X^2$ , can be selected from the group consisting of O, S, NH, NR,  $[NRR'+]$   $NRR'^+$ , CO, COO, COS, S-S, SO, SO<sub>2</sub>, CO-NR', CS-NR', Si-O, and aryl or diaryl groups or  $X^1-X^4$  may be absent;

$B^1$  and  $B^2$ , independently of one another, are optional groups selected from COO, CO, CO-NR', CS-NR',  $(CH_2)_q-CONR'$ ,  $(CH_2)_q-CS-NR'$ , or  $(CH_2)_q$ ;

n, m, p, q and x are whole numbers that can take values from 0 to about 100, where the sum of  $n+xm+p+q$  is less than about 100;

R is an alkyl, alkenyl, alkynyl, alkoxy or an aryl group that is optionally substituted with one or more alkyl, alkenyl, alkynyl, or alkoxy groups; and

R' is a hydrogen, an alkyl, alkenyl, alkynyl, alkoxy or an aryl group that is optionally substituted with one or more alkyl, alkenyl, alkynyl, or alkoxy groups

wherein one or more of the CH<sub>2</sub> groups in the linker can be optionally substituted with alkyl, alkenyl, alkoxy groups, an aryl group that is optionally substituted with one or more alkyl, alkenyl, alkynyl, or alkoxy groups, an acidic group, a basic group or a group carrying a permanent positive or negative charge; wherein one or more single bonds linking non-adjacent [CH<sub>2</sub>] CH<sub>2</sub> groups in the linker can be replaced with a double or a triple bond and wherein one or more of the atoms in the linker can be substituted with a stable isotope.

8. (Once amended) The reagent of claim 1 wherein the affinity label is selected from the group consisting of a 1,2-diol, glutathione, maltose, a nitrilotriacetic acid group, [or] and an oligohistidine.
13. (Once amended) The reagent of claim 1 wherein PRG is selected from the groups consisting of a amine reactive pentafluorophenyl ester group, an amine reactive N-hydroxy succinimide ester group, sulfonyl halide, isocyanate, isothiocyanate, active ester, tetrafluorophenyl ester, an acid halide, and an acid anhydride; a homoserine lactone reactive primary amine group, and [an] a carboxylic acid reactive amine, alcohols or 2,3,5,6-tetrafluorophenyl trifluoroacetate.
15. (Once amended) The reagent of claim 1 wherein PRG is a substrate for an enzyme [the] a deficiency of which is associated with a birth defect.
16. (Once amended) The reagent of claim 1 wherein PRG is a substrate for an enzyme [the] a deficiency of which is associated with a lysosomal storage disease.
17. (Once amended) The reagent of claim 1 wherein PRG is a substrate for acid sphingomyelinase, galactocerebroside β-galactosidase, β-galactosidase, acetyl-α-D-

glucosaminidase, heparan sulfamidase, acetyl-CoA- $\alpha$ -D-glucosaminide N-acetyltransferase or N-acetylglucosamine-6-sulfatase.

18. (Once amended) The reagent of claim 1 wherein at least one of [B1 or B2] B<sup>1</sup> or B<sup>2</sup> is CO-NR' or CS-NR.

Serial Number: 09/839,884

Amended Specification - Version with markings to show changes made.

Page 1, second full paragraph under the heading CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a division of U.S. patent application serial no. [09/388,062] 09/383,062, filed August 25, 1999, which takes priority under 35 U.S.C. §119(e) from U.S. provisional application serial no. 60/097,788, filed August 25, 1998, and serial no. 60/099,113, filed September 3, 1998, all of which are incorporated in their entirety by reference herein.

Page 2, second full paragraph

At present no protein analytical technology approaches the throughput and level of automation of genomic technology. The most common implementation of proteome analysis is based on the separation of complex protein samples most commonly by two-dimensional gel electrophoresis (2DE) and the subsequent sequential identification of the separated protein species (Ducret et al., 1998; Garrels et al., 1997; Link et al., 1997; Shevchenko et al., 1996; Gygi et al. 1999; Boucherie et al., 1996). This approach has been revolutionized by the development of powerful mass spectrometric techniques and the development of computer algorithms which correlate protein and peptide mass spectral data with sequence databases and thus rapidly and conclusively identify proteins (Eng et al., 1994; Mann and Wilm, 1994; Yates et al., 1995). This technology has reached a level of sensitivity which now permits the identification of essentially any protein which is detectable by conventional protein staining methods including silver staining (Figeys and Aebersold, 1998; Figeys et al., 1996; Figeys et al., 1997; Shevchenko et al., 1996). However, the sequential manner in which samples are processed limits the sample throughput, the most sensitive methods have been difficult to automate and low abundance proteins, such as regulatory proteins, escape detection without prior enrichment, thus effectively limiting the dynamic range of the technique. In the 2DE/(MS)[N]<sup>n</sup> method, proteins are quantified by densitometry of stained spots in the 2DE gels.

Paragraph bridging pages 2 and 3

The development of methods and instrumentation for automated, data-dependent electrospray ionization (ESI) tandem mass spectrometry (MS<sup>n</sup>) in conjunction with microcapillary liquid chromatography (μLC) and database searching has significantly increased the sensitivity and speed of the identification of gel-separated proteins. As an alternative to the 2DE/MS<sup>n</sup>

approach to proteome analysis, the direct analysis by tandem mass spectrometry of peptide mixtures generated by the digestion of complex protein mixtures has been proposed (Dongre et al., 1997).  $\mu$ LC-MS/MS has also been used successfully for the large-scale identification of individual proteins directly from mixtures without gel electrophoretic separation (Link et al., 1999; Opitz et al., 1997). While these approaches dramatically accelerate protein identification, the quantities of the analyzed proteins cannot be easily determined, and these methods have not been shown to substantially alleviate the dynamic range problem also encountered by the 2DE/MS/MS approach. Therefore, low abundance proteins in complex samples are also difficult to analyze by the  $\mu$ LC/MS/MS method without their prior enrichment.

Page 5, second full paragraph

In general, the affinity labeled protein reactive reagents of this invention have three portions: an affinity label (A) covalently linked to a protein reactive group (PRG) through a linker group (L):

#### **A-L-PRG**

The linker may be differentially isotopically labeled, e.g., by substitution of one or more atoms in the linker with a stable isotope thereof. For example, hydrogens can be substituted with deuteriums or [ $C^{12}$  with  $C^{13}$ ]  $^{12}C$  with  $^{13}C$ .

Paragraph bridging pages 5 and 6

The affinity label A functions as a molecular handle that selectively binds covalently or non-covalently, to a capture reagent (CR). Binding to CR facilitates isolation of peptides, substrates or reaction products tagged or labeled with A. In specific embodiments, A is a streptavidin or avidin[n]. After affinity isolation of affinity tagged materials, some of which may be isotopically labeled, the interaction between A and the capture reagent is disrupted or broken to allow MS analysis of the isolated materials. The affinity label may be displaced from the capture reagent by addition of displacing ligand, which may be free A or a derivative of A, or by changing solvent (e.g., solvent type or pH) or temperature conditions or the linker may be cleaved chemically, enzymatically, thermally or photochemically to release the isolated materials for MS analysis.

Paragraph bridging pages 8 and 9

Quantitative relative amounts of proteins in one or more different samples containing protein mixtures (e.g., biological fluids, cell or tissue lysates, etc.) can be determined using

chemically identical, affinity tagged and differentially isotopically labeled reagents to affinity tag and differentially isotopically label proteins in the different samples. In this method, each sample to be compared is treated with a different isotopically labeled reagent to tag certain proteins therein with the affinity label. The treated samples are then combined, preferably in equal amounts, and the proteins in the combined sample are enzymatically digested, if necessary, to generate peptides. Some of the peptides are affinity tagged and in addition tagged peptides originating from different samples are differentially isotopically labeled. As described above, affinity labeled peptides are isolated, released from the capture reagent and analyzed by (LC/MS). Peptides characteristic of their protein origin are sequenced using MS<sup>n</sup> techniques allowing identification of proteins in the samples. The relative amounts of a given protein in each sample is determined by comparing relative abundance of the ions generated from any differentially labeled peptides originating from that protein. The method can be used to assess relative amounts of known proteins in different samples. Further, since the method does not require any prior knowledge of the type of proteins that may be present in the samples, it can be used to identify proteins which are present at different levels in the samples examined. More specifically, the method can be applied to screen for and identify proteins which exhibit differential expression in cells, tissue or biological fluids. It is also possible to determine the absolute amounts of specific proteins in a complex mixture. In this case, a known amount of internal standard, one for each specific protein in the mixture to be quantified, is added to the sample to be analyzed. The internal standard is an affinity tagged peptide that is identical in chemical structure to the affinity tagged peptide to be quantified except that the internal standard is differentially isotopically labeled, either in the peptide or in the affinity tag portion, to distinguish it from the affinity tagged peptide to be quantified. The internal standard can be provided in the sample to be analyzed in other ways. For example, a specific protein or set of proteins can be chemically tagged with an isotopically-labeled affinity tagging reagent. A known amount of this material can be added to the sample to be analyzed. Alternatively, a specific protein or set of proteins may be labeled with heavy atom isotopes and then derivatized with an affinity tagging reagent.

Paragraph bridging pages 11 and 12

Suitable affinity tags bind selectively either covalently or non-covalently and with high affinity to a capture reagent (CR). The CR-A interaction or bond should remain intact after extensive and multiple washings with a variety of solutions to remove non-specifically bound components. The affinity tag binds minimally or preferably not at all to components in the assay

system, except CR, and does not significantly bind to surfaces of reaction vessels. Any non-specific interaction of the affinity tag with other components or surfaces should be disrupted by multiple washes that leave CR-A intact. Further, it must be possible to disrupt the interaction of A and CR to release peptides, substrates or reaction products, for example, by addition of a displacing ligand or by changing the temperature or solvent conditions. Preferably, neither CR [or] nor A react chemically with other components in the assay system and both groups should be chemically stable over the time period of an assay or experiment. The affinity tag preferably does not undergo peptide-like fragmentation during (MS)<sup>n</sup> analysis. The affinity label is preferably soluble in the sample liquid to be analyzed and the CR should remain soluble in the sample liquid even though attached to an insoluble resin such as Agarose. In the case of CR the term soluble means that CR is sufficiently hydrated or otherwise solvated such that it functions properly for binding to A. CR or CR-containing conjugates should not be present in the sample to be analyzed, except when added to capture A.

Page 12, first full paragraph

Examples of A and CR pairs include:

d-biotin or structurally modified biotin-based reagents, including d-iminobiotin, which bind to proteins of [the] avidin/streptavidin, which may, for example, be used in the forms of strepavidin-Agarose, oligomeric-avidin-Agarose, or monomeric-avidin-Agarose;

any 1,2-diol, such as 1,2-dihydroxyethane (HO-CH<sub>2</sub>-CH<sub>2</sub>-OH), and other 1,2-dihydroxyalkanes including those of cyclic alkanes, e.g., 1,2-dihydroxycyclohexane which bind to an alkyl or aryl boronic acid or boronic acid esters, such as phenyl-B(OH)<sub>2</sub> or hexyl-B(OEthyl)<sub>2</sub> which may be attached via the alkyl or aryl group to a solid support material, such as Agarose;

maltose which binds to maltose binding protein (as well as any other sugar/sugar binding protein pair or more generally to any ligand/ligand binding protein pairs that has properties discussed above);

a hapten, such as dinitrophenyl group, for any antibody where the hapten binds to an anti-hapten antibody that recognizes the hapten, for example the dinitrophenyl group will bind to an anti-dinitrophenyl-IgG;

a ligand which binds to a transition metal, for example, an oligomeric histidine will bind to Ni(II), the transition metal CR may be used in the form of a resin bound chelated transition metal, such as nitrilotriacetic acid-chelated Ni(II) or iminodiacetic acid-chelated Ni(II);

glutathione which binds to glutathione-S-transferase.



Page 13, first full paragraph

A displacement ligand, DL, is optionally used to displace A from CR. Suitable DLs are not typically present in samples unless added. DL should be chemically and enzymatically stable in the sample to be analyzed and should not react with or bind to components (other than CR) in samples or bind non-specifically to reaction vessel walls. DL preferably does not undergo peptide-like fragmentation during MS analysis, and its presence in a sample should not significantly suppress the ionization of tagged peptide, substrate or reaction product conjugates.

Page 16, first full paragraph

The requirements discussed above for A, L, PRG, extend to the corresponding [to the] segments of A-L-PRG and the reaction products generated with this reagent.

Page 16, second full paragraph

Internal standards, which are appropriately isotopically labeled, may be employed in the methods of this invention to measure absolute quantitative amounts of proteins in samples. Internal standards are of particular use in assays intended to quantitate affinity tagged products of enzymatic reactions. In this application, the internal standard is chemically identical to the tagged enzymatic product generated by the action of the enzyme on the affinity tagged enzyme substrate, but carries isotope labels which may include  $^2\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ , or  $^{34}\text{S}$ , that allow it to be independently detected by MS techniques. Internal standards for use in the method herein to [quantitative] quantitate one or several proteins in a sample are prepared by reaction of affinity labeled protein reactive reagents with a known protein to generate the affinity tagged peptides generated from digestion of the tagged protein. Affinity tagged peptides internal standards are substantially chemically identical to the corresponding affinity tagged peptides generated from digestion of affinity tagged protein, except that they are differentially isotopically labeled to allow their independent detection by MS techniques.

Page 18, third full paragraph

Protein digestion. The proteins in the sample mixture are digested, typically with trypsin. Alternative proteases are also compatible with the procedure as in fact are chemical fragmentation procedures. In cases in which the preceding steps were performed in the presence of high concentrations of denaturing solubilizing agents the sample mixture [are] is diluted until the denaturant concentration is compatible with the activity of the proteases used.

This step may be omitting in the analysis of small proteins;

Page 19, third full paragraph

Results of [this] applying this method using the biotinylated sulfhydryl reagent and to the quantitative analysis of synthetic peptide samples, to the relative quantitation of the peptides in a protein digest [an] and the tandem mass spectral analysis of a derivatized peptide are shown in Fig. 1, Table 1, and Fig. 2, respectively.

Page 22, first full paragraph

The protein reactive affinity reagent strategy was applied to study differences in steady-state protein expression in the yeast, *S. cerevisiae*, in two non-glucose repressed states (Table 3). Cells were harvested from yeast growing in log-phase utilizing either 2% galactose or 2% ethanol as the carbon source. One-hundred  $\mu\text{g}$  of soluble yeast protein from each cell state were labeled independently with the isotopically different affinity tagged reagents. The labeled samples were combined and subjected to the strategy described in [Fig.] Scheme 1. One fiftieth (the equivalent of approximately 2  $\mu\text{g}$  of protein from each cell state) of the sample was analyzed.

Paragraph bridging pages 23 and 24

The method as applied using a sulfhydryl reactive reagent significantly reduces the complexity of the peptide mixtures because affinity tagged cysteine-containing peptides are selectively isolated. For example, a theoretical tryptic digest of the entire yeast proteome (6113 proteins) produces 344,855 peptides, but only 30,619 of these peptides contain a cysteinyl residue. Thus, the complexity of the mixture is reduced, while protein quantitation and identification are still achieved. The chemical reaction [in] of the sulfhydryl reagent with protein can be performed in the presence of urea, sodium dodecyl sulfate (SDS), salts and other chemicals that do not contain a reactive thiol group. Therefore, proteins can be kept in solution with powerful stabilizing agents until they are enzymatically digested. The sensitivity of the  $\mu\text{LC-MS}^n$  system is dependent of the sample quality. In particular, commonly used protein solubilizing agents are poorly compatible or incompatible with MS. Affinity purification of the tagged peptides completely eliminates contaminants incompatible with MS. The quantitation and identification of low abundance proteins by conventional methods requires large amounts (milligrams) of starting protein lysate and involves some type of enrichment for these low abundance proteins. Assays described above, start with about 100  $\mu\text{g}$  of protein and used no

fractionation techniques. Of this, approximately 1/50 of the protein was analyzed in a single  $\mu$ LC-MS<sup>n</sup> experiment. This system has a limit of detection of 10-20 fmol per peptide (Gygi, S.P. et al. (1999)). For this reason, in the assays described which employ  $\mu$ LC-MS<sup>n</sup> only abundant proteins are detected. However, the methods of this invention are compatible with any biochemical, immunological or cell biological fractionation methods that reduce the mixture complexity and enrich for proteins of low abundance while quantitation is maintained. This method can be redundant in both quantitation and identification if multiple cysteines are detected. There is a dynamic range associated with the ability of the method to quantitate differences in expression levels of affinity tagged peptides which is dependent on both the intensity of the peaks corresponding the peptide pair (or set) and the overall mixture complexity. In addition, this dynamic range will be different for each type of mass spectrometer used. The ion trap was employed in assays described herein because of its ability to collect impressive amounts of sequencing information (thousands of proteins can potentially be identified) in a data-dependent fashion even though it offers a more limited dynamic quantitation range. The dynamic range of the ion trap (based on signal-to-noise ratios) varied depending on the signal intensity of the peptide pair and complexity of the mixture, but differences of up to 100-fold were generally detectable and even larger differences could be determined for more abundant peptides. In addition, protein expression level changes of more than 100-200-fold still identify those proteins as major potential contributors to the phenotypic differences between the two original cell states. The method can be extended to include reactivity toward other functional groups. A small percentage of proteins (8% for *S. cerevisiae*) contain no cysteinyl residues and are therefore missed by analysis using reagents with sulfhydryl group specificity (i.e., thiol group specificity). Affinity tagged reagents with specificities toward functional groups other than sulfhydryl groups will also make cysteine-free proteins susceptible to analysis.

Page 25, fifth full paragraph

Amino-reactive, differentially isotopically labeled affinity tagged reagents are used to identify the N-terminal ion series in MS<sup>n</sup> spectra. In a preferred version of this application, the peptides to be analyzed are derivatized with a 50:50 mixture of an isotopically light and heavy reagent which is specific for amino groups. Fragmentation of the peptides by CID therefore [produce] produces two N-terminal ion series which differ in mass precisely by the mass differential of the reagent species used. This application dramatically reduces the difficulty in determining the amino acid sequence of the derivatized peptide.

Page 28, first full paragraph

P69SV40T cells ( $1 \times 10^7$ ) are biotinylated with an isotopically heavy biotin tagged amino reactive reagent and the M12 cells ( $1 \times 10^7$ ) are biotinylated with a corresponding isotopically light amine reactive biotin tagged amino reactive reagent. IGF-1R is then immunoprecipitated from the combined lysate of both cell lines using an antibody against human IGF-1R and the total mass of immunoprecipitated proteins is digested with trypsin. Trypsin is then neutralized, e.g., by the addition of inhibitors and tagged peptides are purified by biotin-avidin affinity chromatography. The eluted peptides are analyzed by LC-MS and LC-MS<sup>(N)</sup> for peptide quantitation and identification, respectively, as has been described above. Quantitation in this experiment is facilitated by the option to use selective ion monitoring in the MS. In this mode only the masses of tagged peptide ions expected to derive from IGF-1R need be monitored.

Page 28, second full paragraph

The described technique can be applied to compare the differences in relative abundance of cell surface proteins between parental prostate cell line (P69SV40T) and M12 cells to detect and identify those cell surface proteins whose expression level is different in the two cell lines and which may be characteristic of the different cell states. Using the methods described [herein] herein, global, relative quantitation of the cell surface proteins in any two or more cell lines can be analyzed to detect and identify those cell surface proteins characteristic of the different cell states. Results can be [independent] independently confirmed using procedure such as 1D or 2D gels, if applicable, or quantitative western blotting to confirm quantitation results.

Page 29, heading after second full paragraph

Synthesis of affinity tagged protein[s] reactive reagents that are selective for certain protein groups

Page 29, third full paragraph

Synthetic routes of exemplary affinity tagged reagents suitable for use in the methods of this invention are provided in Schemes 2-3 where well-known synthetic techniques are employed in synthesis of the non-deuterated and deuterated reagents.

Paragraph bridging pages 29 and 30

Biotinyl-iodoacetamidyl-4,7,10 trioxatridecanediamine **4** (Scheme 3) consists of a biotin group, a chemically inert spacer [of] capable of being isotopically labeled with stable isotopes and [a] an iodoacetamidyl group, respectively. The biotin group is used for affinity enrichment of peptides derivatized with the reagent, the ethylene glycol linker is differentially isotopically labeled for mass spectral analysis and the iodoacetamidyl group provides specificity of the reagent for sulfhydryl-containing peptides. The reagent [an] can be synthesized in an all hydrogen form (isotopically light form) [with and] with 1-20, and preferably 4-8 deuterium atoms in the linker (isotopically heavy forms).

Page 30, second full paragraph

A feature of the method of this invention as applied to enzyme assays is the use of electrospray ionization mass spectrometry (ESI-MS) (Cole et al., 1997) for the simultaneous detection of enzymatic products and chemically identical internal standards, which are distinguished by stable isotope (deuterium) labeling. A second feature is the use of affinity tagged reagents containing an enzyme substrate which when combined with affinity purification [provide] provides for facile capture of enzymatic products from crude biological fluids. The affinity tagged reagents are designed to contain a target substrate for an enzyme of interest that is covalently attached to an affinity tag via a linker. Action of the enzyme of interest on the substrate conjugate causes cleavage or other modification that changes its molecular mass (Scheme 4). The change of mass is detected by ESI-MS. The linker and affinity tag used preferably facilitate ionization by ESI, block action of other enzymes in the biological fluid, and allow highly selective capture from the complex matrix for facile purification.

Page 31, first full paragraph

An example of this approach is the design and synthesis of affinity tagged enzyme substrate reagents **1** and **2** (Scheme 5) to simultaneously assay lysosomal  $\beta$ -galactosidase and N-acetyl- $\alpha$ -D-glucosaminidase, respectively. Deficiency of the former enzyme results in one of the lysosomal storage diseases, GM<sub>1</sub>-gangliosidosis, a condition that occurs in the population with a frequency of about 1 in 50,000 and leads to early death of affected children.[.] Deficiency of [N-acetyl-R-D-glucosaminidase] N-acetyl- $\alpha$ -D-glucosaminidase results in the rare lysosomal storage disorder Sanfilippo syndrome type B. This example has been described in Gerber et al.

(1999) J. Amer. Chem. Soc. **121**: 1102-1103 which is incorporated by reference herein in its entirety.

Paragraph bridging pages 31 and 32

In addition, the linker is hydrophilic to ensure good water solubility of the substrate conjugate, and it has basic groups which are efficiently protonated by ESI and thus ensure sensitive detection by mass spectrometry. The target carbohydrate substrates are attached to the polyether linker by a  $\beta$ -alanine unit (Scheme 5). The enzymatic product conjugates **3** and **4** are also shown Scheme 5. Conjugates **1** and **2** were prepared as shown in Scheme 5. All reagents were purified to homogeneity by reverse-phase HPLC and characterized by high-field [1 H NMR] 1H-NMR and ESI-MS. The substrate was linked to the diamine spacer by Michael addition of the latter onto the p-acryloylamidophenyl glycoside, (Romanowska et al., 1994) and the intermediate was coupled with the tetrafluorophenyl ester of N-biotinylsarcosine (Wilbur et al., 1997).

Paragraph bridging pages 33 and 34

The approach described for assaying enzymes using substrate reagents and ESI-MS can be broadly applied. The multiplex technique can be expanded to assay dozens or more enzymes simultaneously in a single reaction, obviating the need for multiple assays to assist in confirming diagnoses of rare disorders. The method can be used to measure several enzymes simultaneously when evaluating the rate of chemical flux through a specific biochemical pathway or for monitoring biochemical signaling pathways. The affinity tag-capture reagent method for isolation of affinity tagged reaction products and substrates from complex mixtures is technically simple and can be readily automated, particular when biotin-streptavidin is employed. Because of the high sensitivity of the ESI-MS detection employed, which requires only sub-microgram quantities of the substrate reagents per assay, the synthesis of several hundred substrate reagents on a low-gram scale becomes practical and economical. Since most enzyme active sites are exposed to solvent, it is possible to attach an affinity tagged linker to most enzyme substrates while preserving enzymatic activity. Scheme 6 provides the structures of several additional enzyme substrates, suitable for use in this method, indicating by arrows allowable positions for tag attachment sites. Allowable tag sites for additional enzyme substrates can be determined by review of X-ray crystal structures of enzyme-substrate or enzyme-substrate analog structures. Using a standard computer graphics [program ,] program, available X-ray data and by attaching an extended chain butyl group (as a model for the affinity

tagged linker) to potential tag attachment sites, suitable attachment sites that show there are no enzyme-atoms in van der Waals overlap with the model tag can be predicted.

Page 34, second full paragraph

Table 4 provides exemplary enzymes that are [associates] associated with certain birth defects or disease states. These enzymes can be assayed by the methods described herein.

Page 36, first full paragraph

The synthesis of a biotinylated dolichol<sub>10</sub>-substrate conjugate containing a sarcosinyl linker (B-S-Dol<sub>10</sub>-P) is shown in Scheme 9. Protected citronellol (R = t-BuSiMe<sub>2</sub>) is regioselectively oxidized at the terminal allylic methyl group (McMurry and Kocovsky, 1984), and the allylic alcohol is coupled with biotinylsarcosine active ester (R = CH<sub>3</sub>). The citronellol 1-hydroxy group is subsequently deprotected and phosphorylated with POCl<sub>3</sub> (Rush and Wachter, 1995). In a parallel synthesis, d<sub>5</sub>-sarcosine, CD<sub>3</sub>NHCD<sub>2</sub>COOH, is used to prepare the isotopically labeled (heavy) reagent for use as an internal standard. d<sub>5</sub>-Sarcosine is readily [prepares form] prepared from commercially available materials (BrCD<sub>2</sub>COOD and CD<sub>3</sub>NH<sub>2</sub>) using standard synthetic techniques.

Paragraph bridging pages 36 and 37

**CDGS Type II** results from defective GlcNAc transferase II (GlcNAc-T II) which transfers GlcNAc from UDP-GlcNAc to the 2-position of a mannose residue in the intermediate branched oligosaccharide (the Core Region) in the process of building up the disialobiantennary chain (Scheme 10) (Schachter, 1986, Brockhausen et al, 1989). GlcNAc transferase II is one of the six known enzymes that mediate highly regiospecific glycosylation of the mannose residues in the Core Region. The Core Region is anchored at the reducing end to chitobiosylasparagine, where the asparagine residue is part of the peptide chain of the glycosylated protein. The latter structure unit in the substrate can be replaced by a hydrophobic chain without loss of enzyme activity (Kaur et al, 1991). Thus, the substrate conjugate for CDGS Type II is assembled by linking [a] an affinity-labeled linker group to the reducing end [to] of chitobiosylasparagine. However, the latter structure unit in the substrate can be replaced by a hydrophobic chain without loss of enzyme activity (Kaur et al, 1991). For example, commercially available  $\alpha$ -D-manno-pyranosylphenylisothiocyanate can be coupled to a biotin-labeled linker and the 5,6-hydroxyls are selectively protected as illustrated in Scheme 11 (Paulsen and Meinjohanns, 1992). Coupling of the equatorial 3-OH with per-O-acetylmannosyl-

1-trichloroacetamidate (Paulsen et al, 1993) will provide a disaccharide conjugate [(Scheme 12.)] (Scheme 12). If a minor amount of coupling occurs at the axial 2-OH group the products can be separated by HPLC. After deprotection, the primary 6-OH is coupled with a second equivalent of per-O-acetylmannosyl-1-trichloroacetamidate to yield the Core Region conjugate. Deprotection of the O-acetyl groups yields the substrate conjugate for GlcNAc transferase I which can be converted to the GlcNAc-T II substrate by enzymatic glycosyl transfer using a Triton X-100 rabbit liver extract, a reaction that has been carried out on a preparative scale (Kaur et al. 1981).

Page 39, third full paragraph

The total enzyme reaction volume is 20 - 30 mL. The substrate stock solutions are maintained at concentrations of 3mM (SFB) and 2mM (GM1). These concentrations were measured by <sup>1</sup>H-NMR signal integration versus an internal standard (formamide proton of DMF). Final concentration of substrates is 0.3 and 0.2 mM, respectively. A volume of reaction buffer (e.g. 200mM sodium citrate, pH 4.5) equal to the difference of the substrate addition (2-3 mL) plus sufficient cell sample volume to equal 50 [μ] - 75mcg total protein from 20-30 mL is added to a 0.5 mL Eppendorf tube, followed by substrate. The sample is cooled on ice, and patient cell sample is added. The reaction is initiated by incubation at 37°C.

Page 40, fifth full paragraph

Patient skin fibroblasts were obtained as frozen pellets, and stored at [μ]-20°C until use. Two GM1 affected samples and six normal controls were analyzed.

Page 43, first full paragraph

1. 2,3,5,6-Tetrafluorophenyl trifluoroacetate (1) 25g (0.15mol)

2,3,5,6-tetrafluorophenol, 35 mL (0.2mol) trifluoroacetic anhydride and 0.5 mL boron trifluoride etherate were refluxed for 18 hours under argon atmosphere. Trifluoroacetic anhydride and trifluoroacetic acid were removed by distillation at room temperature. The trifluoroacetic anhydride fraction was returned to the mixture, and the reaction was refluxed for 24 hours. This was repeated twice. After final distillation at room temperature, the desired product 1 was distilled at reduced pressure (62°C/45mmHg) to produce a colorless liquid (30g, 82%).

<sup>1</sup>H-NMR. (Gamper, H. B., 1993). [Biotin-2,3,5,6-tetrafluorophenyl ester (2) A 2.5g (10.3mmol) quantity of d-biotin in 20 mL anhydrous DMF under argon atmosphere was warmed to 60°C with stirring to effect dissolution. 1.7 mL (12.5mmol) triethylamine was added, followed by 3.4g



(12.5mmol) 1. The mixture was stirred for 2 hours, after which the solvent was removed by rotary evaporation. The resultant semi-solid was triturated with 15 mL ether twice to produce a white solid (2.6g, 65%). <sup>1</sup>H-NMR. (Wilbur, D. S., et al., 1997). N-methylglycylbiotinamide-methyl ester (3) A 2.5g (6.4mmol) quantity of biotin tetrafluorophenyl ester in 30 mL anhydrous DMF under argon atmosphere was added to a mixture of 1.1g (7.7mmol) N-methylglycine methyl ester hydrochloride dissolved in 10 mL anhydrous DMF and 1.25 mL (9.0 mmol) triethylamine. The reaction mixture was stirred at room temperature for 2 hours, then the solvent was removed by rotary evaporation. The residue was extracted with chloroform (2x100 mL), washed with water (2x20 mL), and dried with anhydrous sodium sulfate. The solvent was removed under vacuum to yield 2.1g (98 %) of methyl ester of N-methylglycine biotinamide as an oil. <sup>1</sup>H-NMR. (Wilbur, D. S., et al., 1997).]

2. Biotin-2,3,5,6-tetrafluorophenyl ester (2) A 2.5g (10.3mmol) quantity of d-biotin in 20 mL anhydrous DMF under argon atmosphere was warmed to 60°C with stirring to effect dissolution. 1.7 mL (12.5mmol) triethylamine was added, followed by 3.4g (12.5mmol) 1. The mixture was stirred for 2 hours, after which the solvent was removed by rotary evaporation. The resultant semi-solid was triturated with 15 mL ether twice to produce a white solid (2.6g, 65%). <sup>1</sup>H-NMR. (Wilbur, D. S., et al., 1997).

3. N-methylglycylbiotinamide-methyl ester (3) A 2.5g (6.4mmol) quantity of biotin tetrafluorophenyl ester in 30 mL anhydrous DMF under argon atmosphere was added to a mixture of 1.1g (7.7mmol) N-methylglycine methyl ester hydrochloride dissolved in 10 mL anhydrous DMF and 1.25 mL (9.0 mmol) triethylamine. The reaction mixture was stirred at room temperature for 2 hours, then the solvent was removed by rotary evaporation. The residue was extracted with chloroform (2x100 mL), washed with water (2x20 mL), and dried with anhydrous sodium sulfate. The solvent was removed under vacuum to yield 2.1g (98 %) of methyl ester of N-methylglycine biotinamide as an oil. <sup>1</sup>H-NMR. (Wilbur, D. S., et al., 1997).

Paragraph bridging pages 44 and 45

7. GM1 substrate conjugate of 4 and 6 (7) A 2.5mg (7.4mcmmol) quantity of 4 was dissolved in 1.5 mL anhydrous DMF with stirring, under argon atmosphere. 5 mL triethylamine was added, followed by 2.3mg (8.8mcmmol) 1. The formation of active ester was monitored by silica TLC (5:1 CHCl<sub>3</sub>/CH<sub>3</sub>OH, R<sub>f</sub> 0.5, UV) by briefly drying the spotted TLC plate with a stream of air. After 25 minutes, the mixture was added to 3.2mg (5.9mcmmol) 6 in 1 mL anhydrous DMF. After 2 hours, the solvent was removed by vacuum centrifugation and the final product was purified

by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: [H<sub>2</sub>O] H<sub>2</sub>O (0.08%TFA)/ ACN (0.08%TFA)). Yield 4.6 mg. (For analogous chemistry, see Wilbur, D. S., et al., 1997).

Page 46, second full paragraph

12. GM1 internal standard conjugate (12) 1.8mg 11 was added to 2 mL of 100mM Tris/10mM [MgCl<sub>2</sub>] MgCl<sub>2</sub>, pH 7.3 buffer with stirring. 15 units recombinant β-D-galactosidase (Sigma) was added, and after 12 hours the mixture was purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H<sub>2</sub>O (0.08%TFA)/ACN (0.08%TFA)). Yield 1.5 mg.

Page 51, third full paragraph

23. SFB internal standard conjugate (29) 1.2mg 28 was added to 2 mL of 100mM Tris/10mM [MgCl<sub>2</sub>] MgCl<sub>2</sub>, pH 7.3 buffer with stirring. 15 units recombinant β-D-galactosidase (Sigma) [was] were added, and after 12 hours the mixture was purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H<sub>2</sub>O (0.08%TFA)/ACN (0.08%TFA)). Yield 0.7 mg.

Page 56, first full paragraph

An automated LC-MS/MS system for the identification of proteins by their amino acid sequence has been developed. A schematic representation is shown in Fig. 7 The system, which consists of an [autosample,] autosampler, a capillary HPLC system connected on-line to an ESI triple quadrupole MS/MS instrument and a data system is operated in the following way: Proteins (typically separated by 1D or 2D gel electrophoresis) are cleaved with a specific protease, usually trypsin. the resulting cleavage fragments are placed in an autosampler. Every 37 minutes the autosampler injects one sample into the HPLC system and the peptides are separated by capillary reverse-phase chromatography. As separated peptides elute from the chromatography column, they are ionized by the ESI process, enter the MS and the mass to charge ratio (m/z) is measured. Any peptide ion whose intensity exceeds a predetermined intensity threshold is automatically selected by the instrument and collided in the collision cell with inert gas. These collisions result in peptide fragmentation, primarily at the bonds of the peptide backbone (collision induced dissociation, CID). The masses of the CID fragments are measured and recorded in the data system. The CID spectrum of a peptide contains sufficient information to identify the protein by searching sequence databases with the uninterpreted

MS/MS spectra. This is accomplished with the Sequest program. The program identifies each peptide in a sequence database which has the same mass as the peptide that was selected in the MS for CID and predicts the MS/MS spectrum for each one of the isobaric peptides. By matching the experimentally determined CID spectrum with computer generated theoretical CID spectra, the protein from which the observed peptide originated is identified. The system is capable of analyzing protein samples in a fully automated fashion at a pace of less than 40 min. per sample. Since each peptide represents an independent protein identification and usually multiple peptides are derived from one protein, protein identification by this method is redundant and tolerant to proteins co-migrating in a gel. The system is well suited for the detection and characterization of modified residues within polypeptide chains. The LC-MS/MS technique and automated analysis of the generated CID spectra can be used for the methods of this invention.

Page 57, first full paragraph

Protein identification by this method is based on the same principle as described above, except that peptide separation and ionization are performed at significantly higher sensitivity. Fig. 8 shows a schematic representation of the key design elements. The design of the system and its mode of operation have been published. Peptides derived from protein digests are concentrated by SPE, separated by CE and analyzed by ESI-MS/MS. The resulting uninterpreted CID spectra are used to search sequence databases with the Sequest software system. The SPE extraction device is a small reversed-phase chromatography column of the dimensions 0.18 x 1 mm which is directly packed in a fused silica separation capillary. Peptides contained in a sample solution are adsorbed and concentrated on the SPE device, eluted in an estimated 100 - 300 nl of organic solvent and further concentrated by electrophoretic stacking and/or isotachopheresis to an estimated volume of 5-30 nl. The peptides are then separated by CE in a 20  $\mu$ m or 50  $\mu$ m i.d. capillary and directly ionized by ESI as they leave the capillary (see reference 13 for design of the microspray ionization source). With this system, peptide masses can be determined at a sensitivity of 660 attomoles (approx. 500 fg for a 20 residue peptide) at a concentration limit of 33 amol/ $\mu$ l and [that] proteins can be identified by the CID spectra of automatically selected peptides at less than 10 fmol (0.5 ng for a protein of 50 kDa) of sample at a concentration limit of less than 300 amol/ $\mu$ l. [this] This technique is used for the analysis at very high sensitivity of the peptide samples generated by the experiments. It has also been demonstrated that the analysis time available for automated CID experiments can be significantly extended by data-dependent modulation of the CE voltage. If several peptide ions are detected coincidentally in the MS, the CE voltage is automatically dropped. This results in a

reduction of the electroosmotic flow out of the capillary and therefore in an extension of the time period available for selecting peptide ions for CID. The net effect of this peak parking technique is an extension of the dynamic range of the technique because the increased time available is used for CID of ions with a low ion [current] current. Once all the peptide ions are analyzed, electrophoresis is automatically reaccelerated by increasing the CE voltage to the original value.

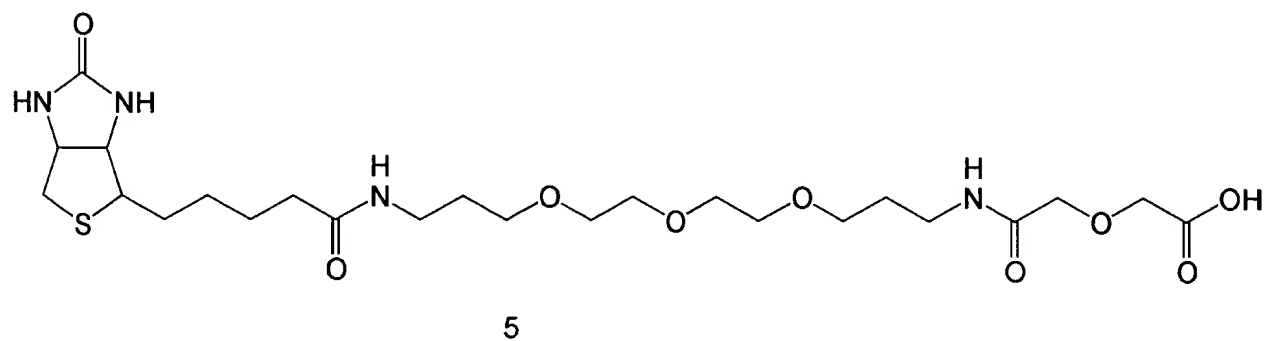
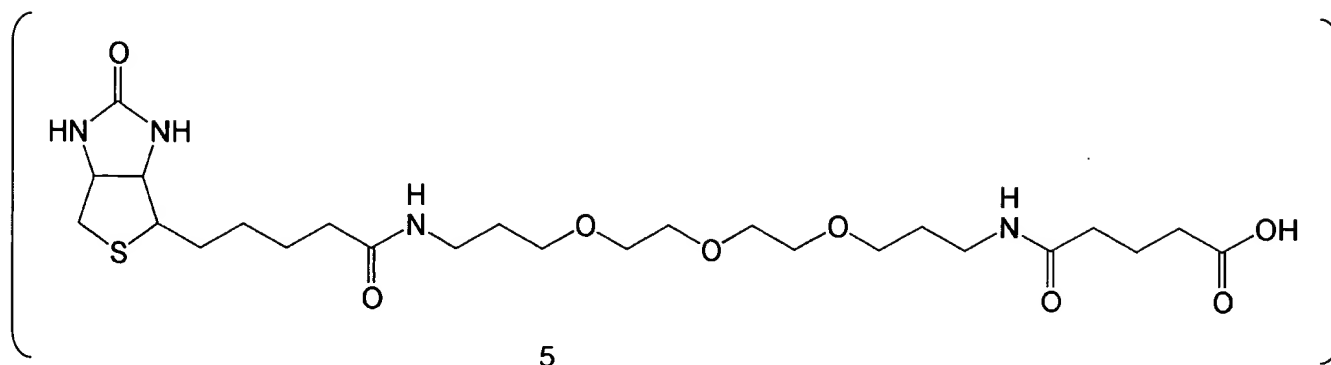
Page 58, replace Table 1 with the following

TABLE 1. Relative, redundant quantitation of  $\alpha$ -lactalbumin abundance (after mixing with known amount of the same protein with cysteines modified with isotopically heavy biotinylating reagent)

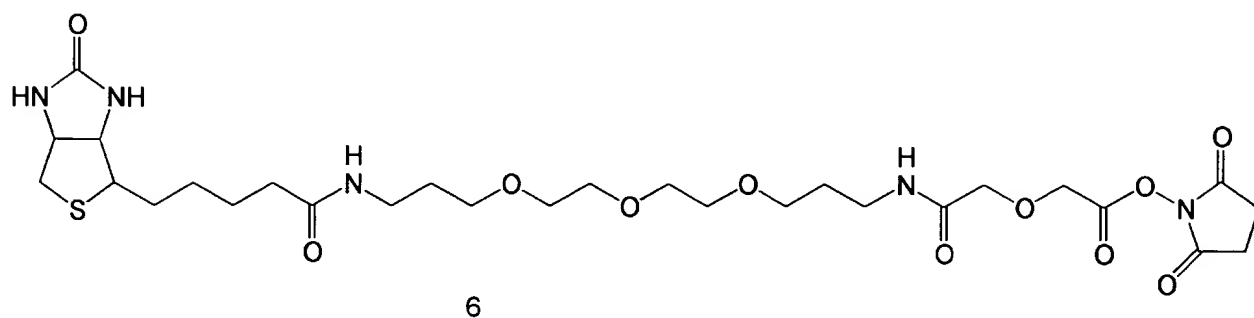
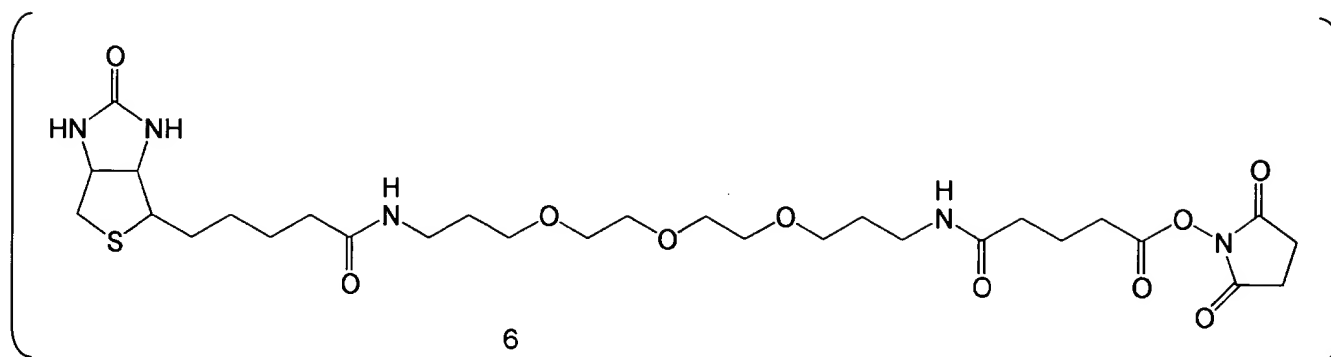
Peptide #	<i>m/z</i> (light)	Charge state	Peptide Sequence	Ratio (heavy:light)
1	518.4	2+	(K) IWCK	2.70
2	568.4	2+	(K) ALCSEK (SEQ ID NO:2)	2.68
3	570.4	2+	(K) CEVFR (SEQ ID NO:3)	2.9
4	760.5	2+	(K) LDQWLCEK (SEQ ID NO:4)	2.82
5	710.1	3+	(K) FLDDDLTDDIMCVK (SEQ ID NO:5)	2.88
6	954.2	3+	(K) DDQNPHSSNICNISCDK (SEQ ID NO:6)	2.9
7	1286.9	4+	(K) GYGGVSLPEWVCTTFHTSGYDT QAIVQNNDSTEYGLFQINN (SEQ ID NO: 7) [(SEQ ID NO.)]	NA <sup>a</sup>

<sup>a</sup> Isotope ratio was not analyzed because on a [4<sup>+</sup>] 4<sup>+</sup> peptide the isotope patterns were highly overlapping due to differences of only 2 amu between heavy and light ions.

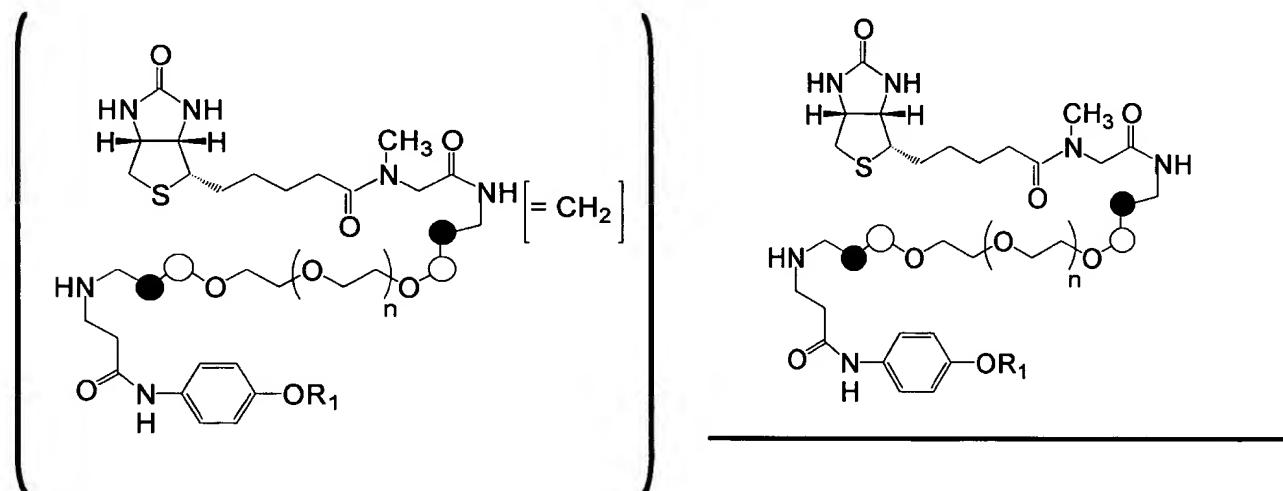
Page 76, replace Scheme 3, Structure 5 with the following



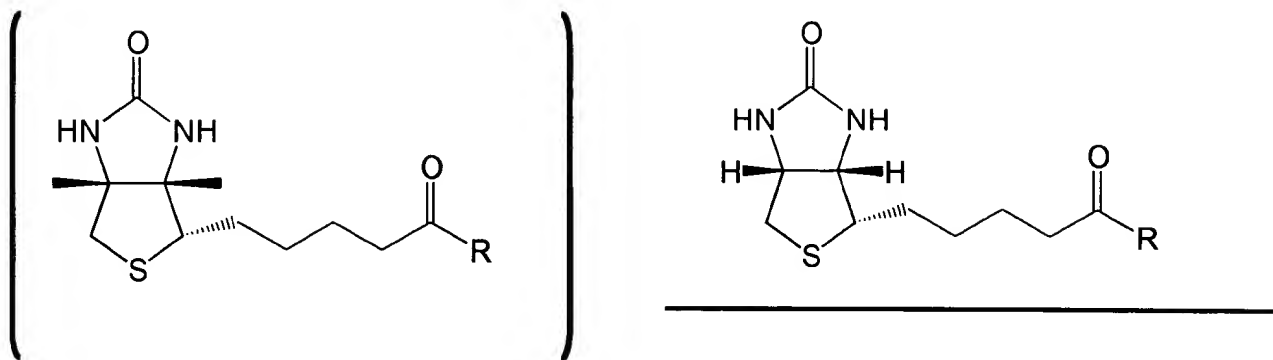
Page 76, replace Scheme 3 structure 6 with the following



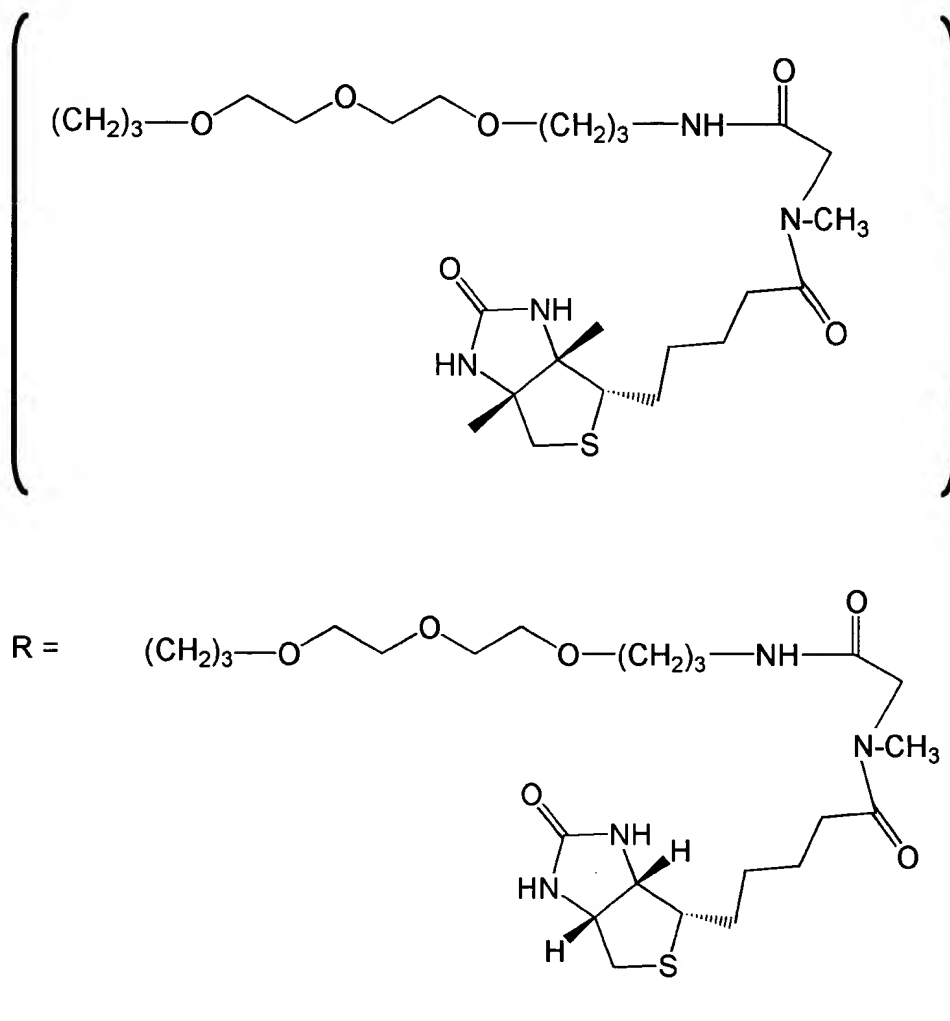
Page 77, replace Scheme 5 with the following



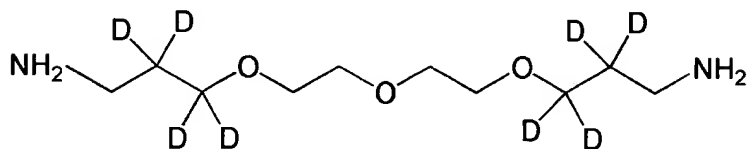
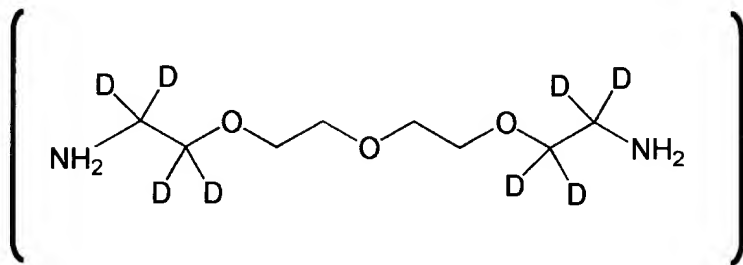
Page 83, replace the chemical structure containing the “R” group in Scheme 14 with the following



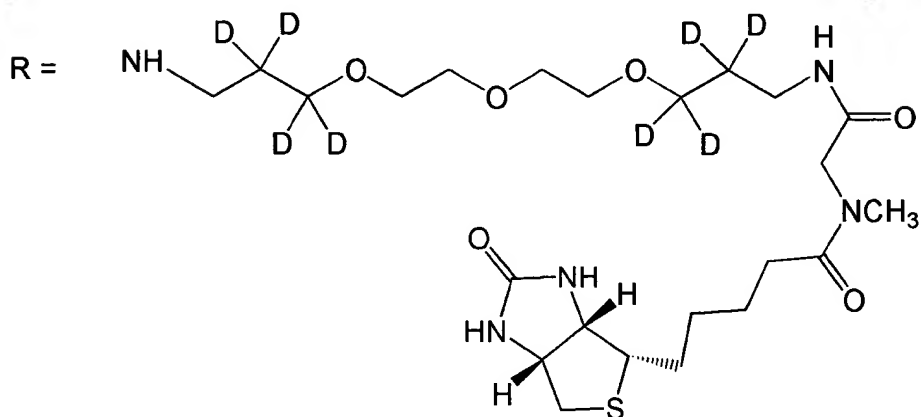
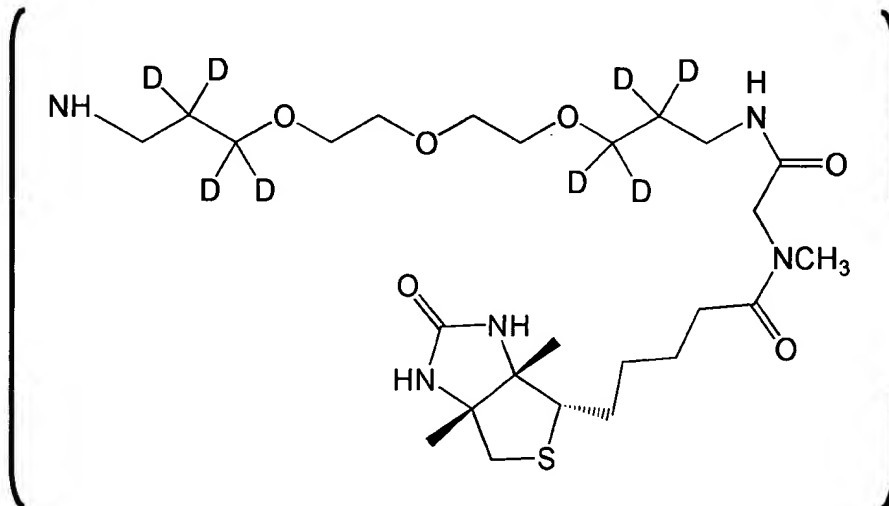
Page 84, replace Scheme 15, structure 7 with the following



Page 84, replace Scheme 15, structure 9 with the following

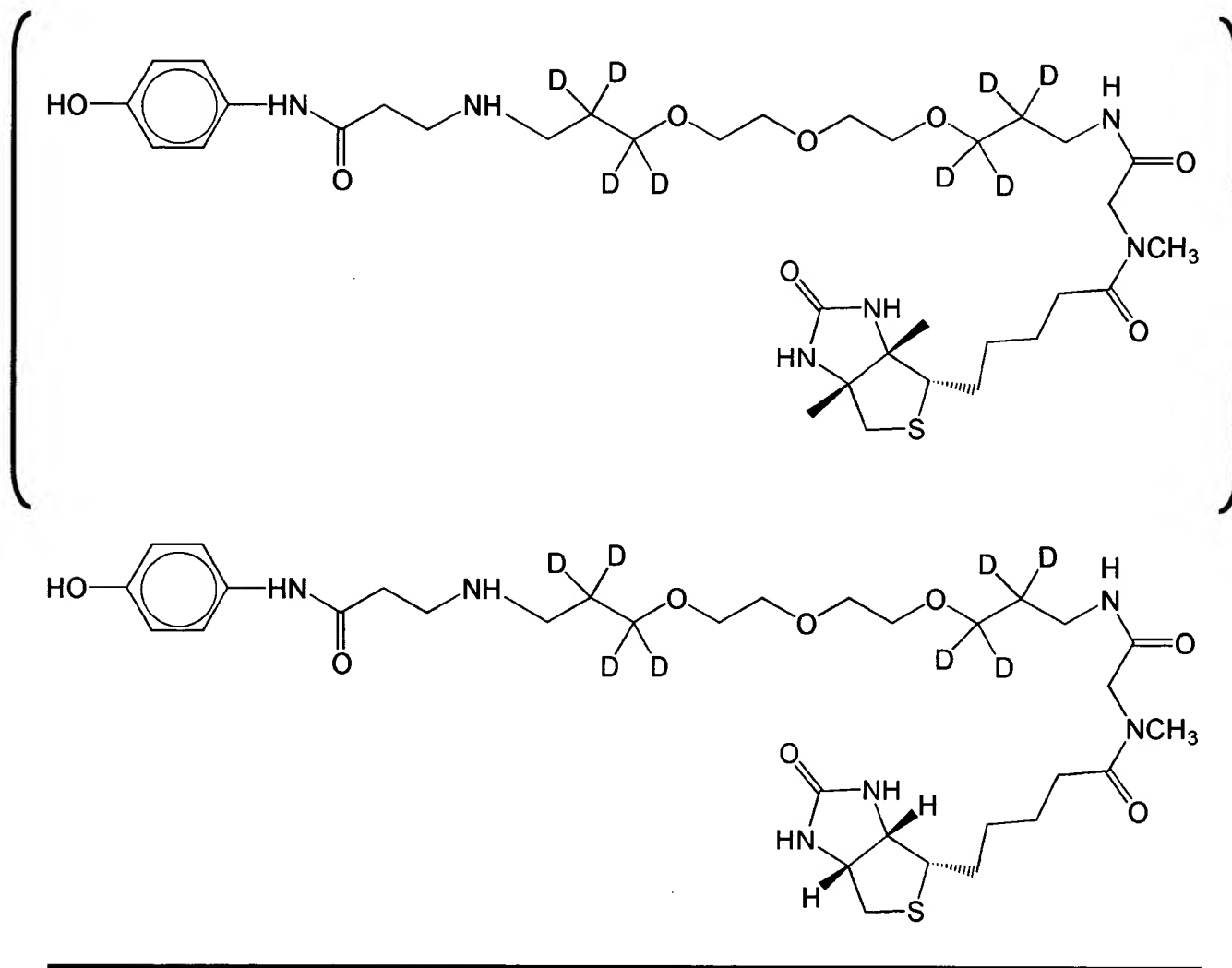


Page 85, replace Scheme 16, structure 11 with the following

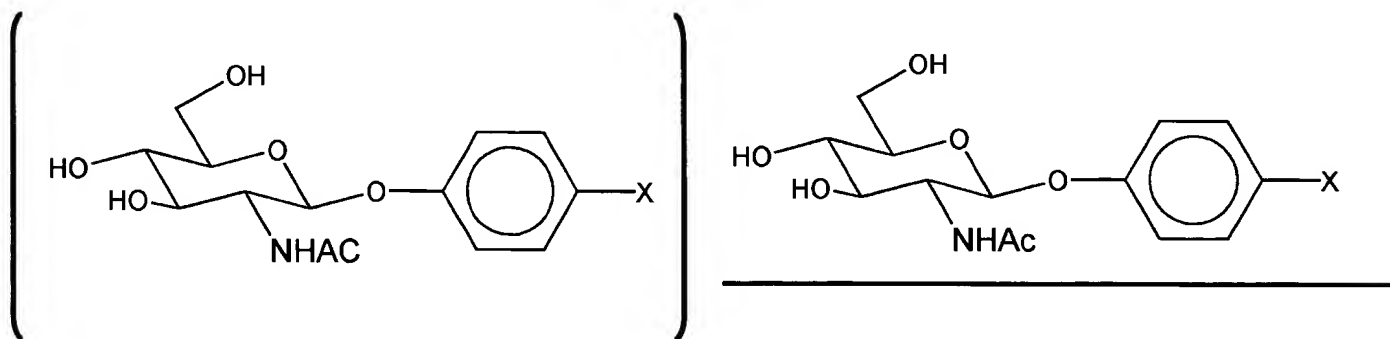




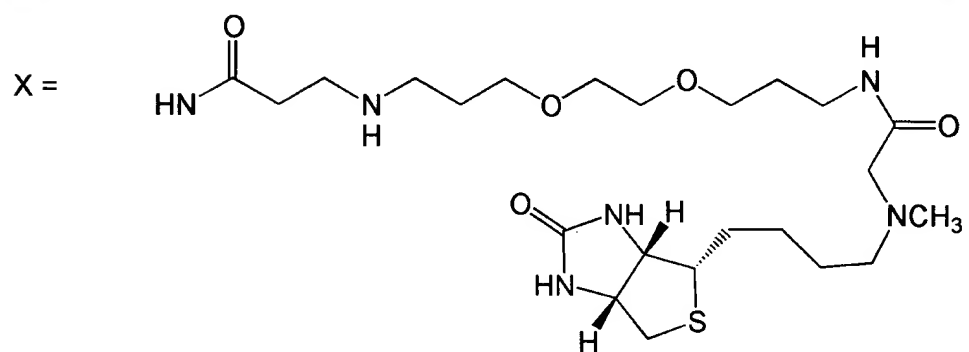
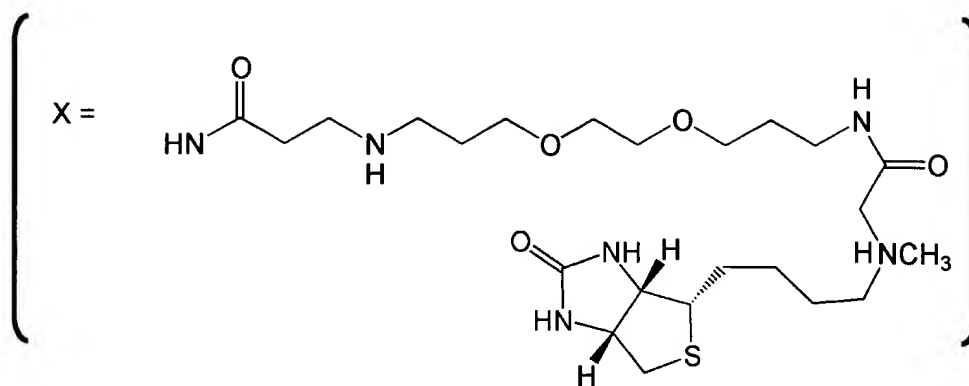
Page 85, replace Scheme 16, structure 12 with the following



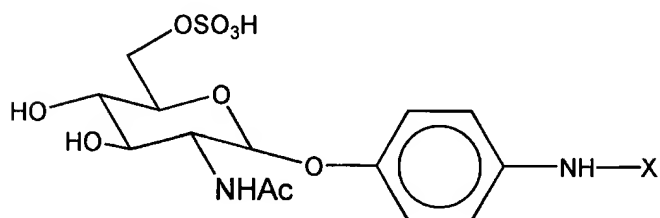
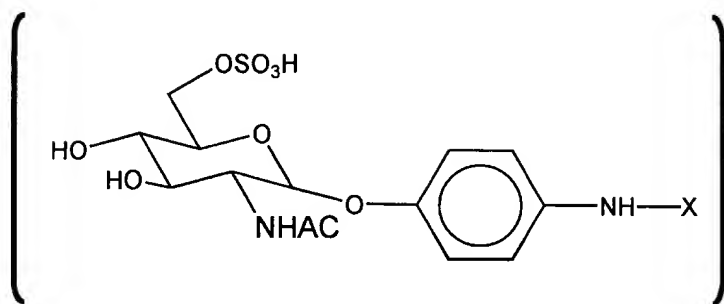
Page 87, replace the first structure in Scheme 18 with the following



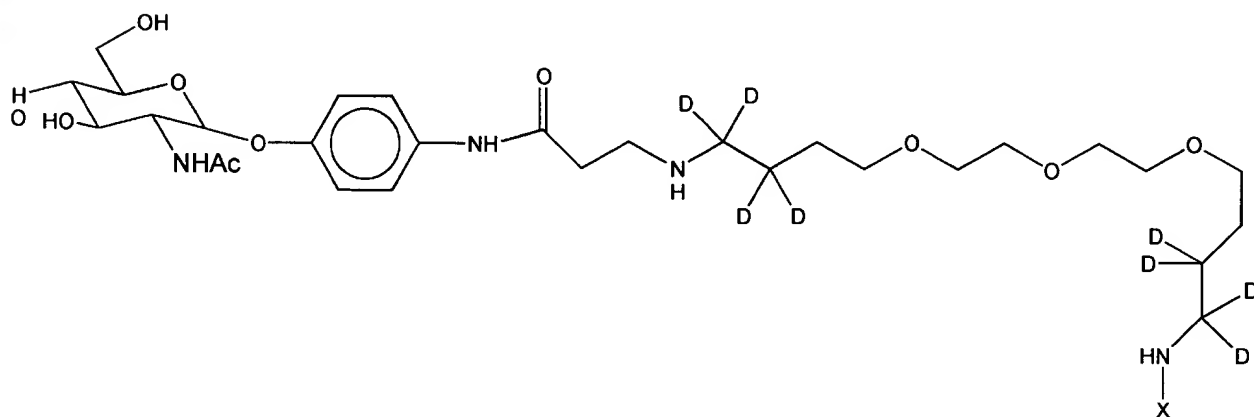
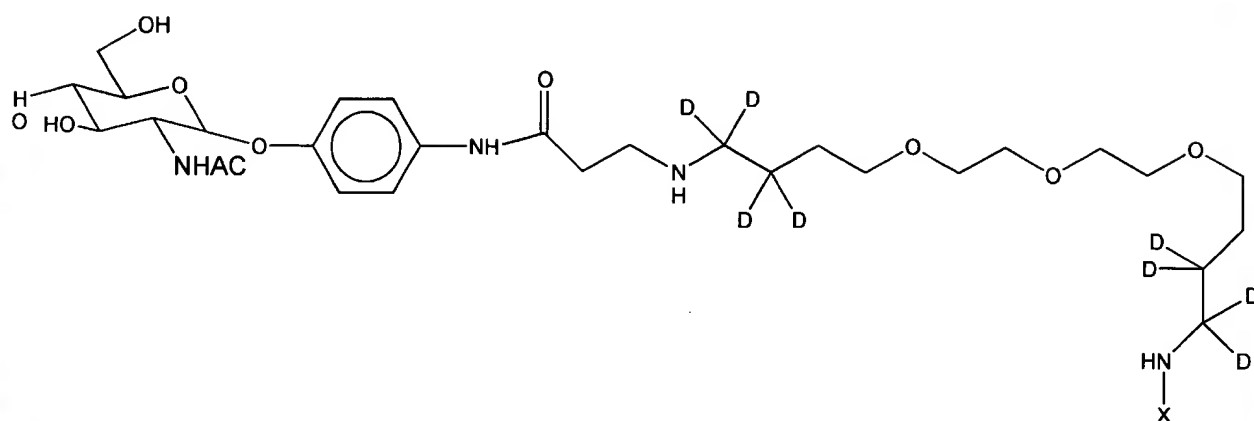
Page 87, replace Scheme 18, structure 24 with the following



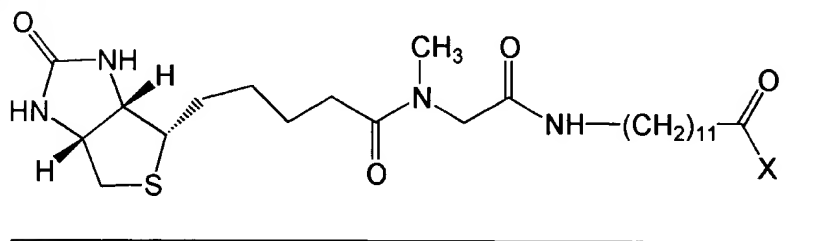
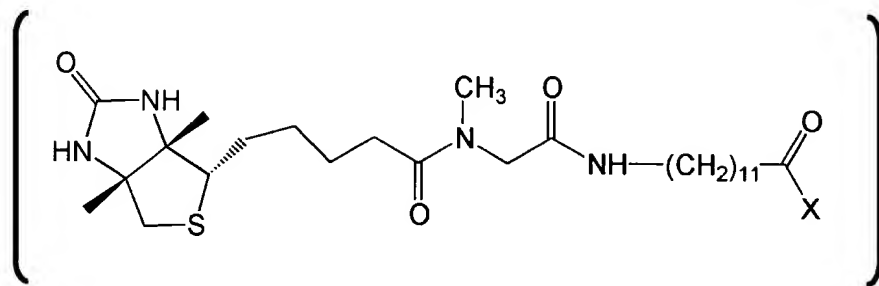
Page 89, replace the first structure in Scheme 20 with the following



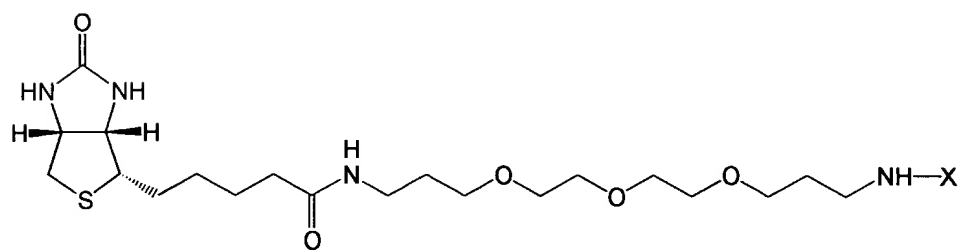
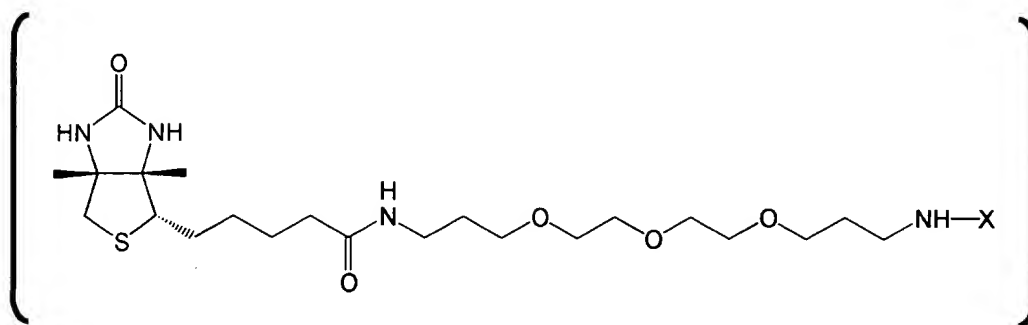
Page 90, replace the chemical structure containing X in Scheme 21 with the following



Page 91, replace the first chemical structure in Scheme 22 with the following



Page 92, replace the first chemical structure containing "X" in Scheme 23 with the following



Page 92, replace the second chemical structure containing "X" in Scheme 23 with the following

